SUPPLEMENTARY APPENDIX

Bafilomycin A1 targets both autophagy and apoptosis pathways in pediatric B-cell acute lymphoblastic leukemia

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Hematology Center of Cyrus Tang Medical Institute, Jiangsu Institute of Hematology, Jiangsu Key Laboratory for Stem Cell Research, Collaborative Innovation Center of Hematology, Affiliated Children's Hospital, Soochow University School of Medicine, Suzhou, China

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The online version of this article has a Supplementary Appendix.
Manuscript received on July 7, 2014. Manuscript accepted on December 5, 2014.
Correspondence: jrwang@suda.edu.cn

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Hematology Center of Cyrus Tang Medical Institute, Jiangsu Institute of Hematology, Jiangsu Key Laboratory for Stem Cell Research, Collaborative Innovation Center of Hematology, Affiliated Children's Hospital, Soochow University School of Medicine, Suzhou, China

‡**Address correspondence to**: Jianrong Wang, Hematology Center of Cyrus Tang Medical Institute, Soochow University School of Medicine, 199 Ren'ai Road, Suzhou 215123, China; Tel/Fax: 86-512-65884331; E-mail address: jrwang@suda.edu.cn

Supplementary Methods

Cell proliferation assay. Cells were collected and resuspended in serum-free 1640 medium at a density of 4×10^6 cells/mL and mixed gently immediately after adding the dye [5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE; Sigma-Aldrich)] into the suspension to a final working concentration of 3 μM, then incubated the mixture in the dark for 15 minutes at 37ºC, after which 5 fold volume of cold 1640 medium containing 10% FBS was added to terminate reactions. The resultant cells were washed twice with culture medium. Finally, cell suspension (stained with CFSE) was dispensed into 24 well plate and added different drugs into corresponding wells. Cells were harvested at appropriate time, washed twice and resuspended with PBS, followed by analysis on a BD Calibur flow cytometer. Cell group not treated with CFSE served as a negative control.

Cell cycle analysis. To analyze cell-cycle status, cells were pelleted by centrifugation for 5 minutes at 200 g, washed in PBS, and resuspended in 70% ice-cold ethanol. Cells were fixed overnight and then stained with propidium iodide. The pretreated cells were analyzed using a BD Calibur Flow Cytometer.

Western blotting, co-immunoprecipitation and antibodies. Primary and secondary antibodies were provided by Cell Signaling Technology (Danvers, MA, USA). Signals were detected with the ECL Plus reagent by Perkin Elmer (Boston, MA, USA). Cell was collected and resuspended in lysis buffer containing protease inhibitor cocktail and PhosSTOP phosphatase

inhibitor cocktail (Roche, USA). The resuspended cell pellet was vortexed for 20 seconds and then incubated on ice for 30 min and centrifuged at 13,000 g for 20 min at 4°C. The supernatants were collected and the protein concentration was determined using the BCA protein assay kit (Thermo, USA) and heated at 95 °C for 5 min. Equal amounts of protein were subjected to SDS-PAGE. Samples were loaded on a polyacrylamide gel for electrophoresis separation and transferred to a polyvinylidenedifluoride membrane (Millipore, USA). Membranes were blocked in 5% non-fat dry-milk and incubated overnight at 4°C with the primary antibodies. The primary antibodies were revealed using an appropriate horseradish peroxidase (HRP)–conjugated secondary antibody and detected by an enhanced chemiluminescence kit (Pierce, USA). For Co-immunoprecipitation, 50 μg of antibody against each bait protein was immobilized in a coupling gel, and each 1 mg of cell lysates prepared from different treatments was incubated with the antibody-immobilized coupling gel using Co-Immunoprecipitation kit (Pierce, USA) following the manufacturer's protocol.

Autophagic flux assay. To induce autophagy, cells were incubated with nutrient-depleted medium (HBSS) for 12 hours. To study autophagic flux, we constructed a 697 stable transfectant leukemia cell line expressing a reporter GFP-LC3, and fluorescence images were taken using Olympus Confocal Microscope and ImageStreamX Mark Ⅱ (Amnis, Seattle, USA) and analyzed with IDEAS 4.0 software by measuring total number of green dots.

Apoptosis assay. Cells were seeded into 24-well plates at a density of 1×10^5 cells/well. After 72 h incubation, cells were assessed by Annexin V staining using Annexin V-FITC apoptosis detection kit (BD Bioscience, USA) followed the manufacturer's instructions and analyzed by flow cytometry using a BD Calibur Flow Cytometer and BD Cell Quest program software (Becton Dickinson, USA).

Small RNA Interference. Expression of AIF in leukemia cell lines was transiently silenced using small interfering RNA (siRNA) of AIF. The sense and antisense strands of AIF-1 siRNA (5-AUGCAGAACUCCAAGCACGTT-3 and 5- CGUGCUUGGAGUUCUGCAUTT-3), AIF-2 (5-GAUCCUCCCCGAAUACCUCTT -3 and 5- GAGGUAUUCGGGGAGGAUCTT-3), the negative control siRNA (5-UUCUCCGAACGUGUCACGUTT-3 and 5-ACGUGACACGUUCGGAGAATT-3) were synthesized by GenePharma company (Shanghai, China). Briefly, cells were transfected with 100 nM siRNA for 48 hours using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After siRNA transfection, AIF mRNA levels were detected by Q-PCR and AIF protein expression levels were detected by western blotting.

Fluorescence microscopy. The B-ALL cells were pelleted by centrifugation for 5 minutes at

200 g, washed in PBS, fixed in 4% paraformaldehyde at RT for 10min. After rinse three times in PBS for 5min each time, the cells were blocked with 5% goat serum in PBS (with 0.3% Triton X-100) for 60 minutes. The cells were incubated first with the primary antibody (diluted in PBS supplemented with 0.3%Triton X-100 and 1% bovine serum albumin, overnight in a humid chamber at 4°C) and then with FITC-conjugated secondary antibodies (diluted 1:1000, for 1h at 37°C in a humid chamber). Excess of unbound antibody was removed at each step by three washes with PBS. The nuclear material was stained with 20 μg/ml Hoechst 33342 (Invitrogen, USA) at room temperature for 10 min. The cells were washed twice with PBS. The images were obtained using an Olympus confocal microscope.

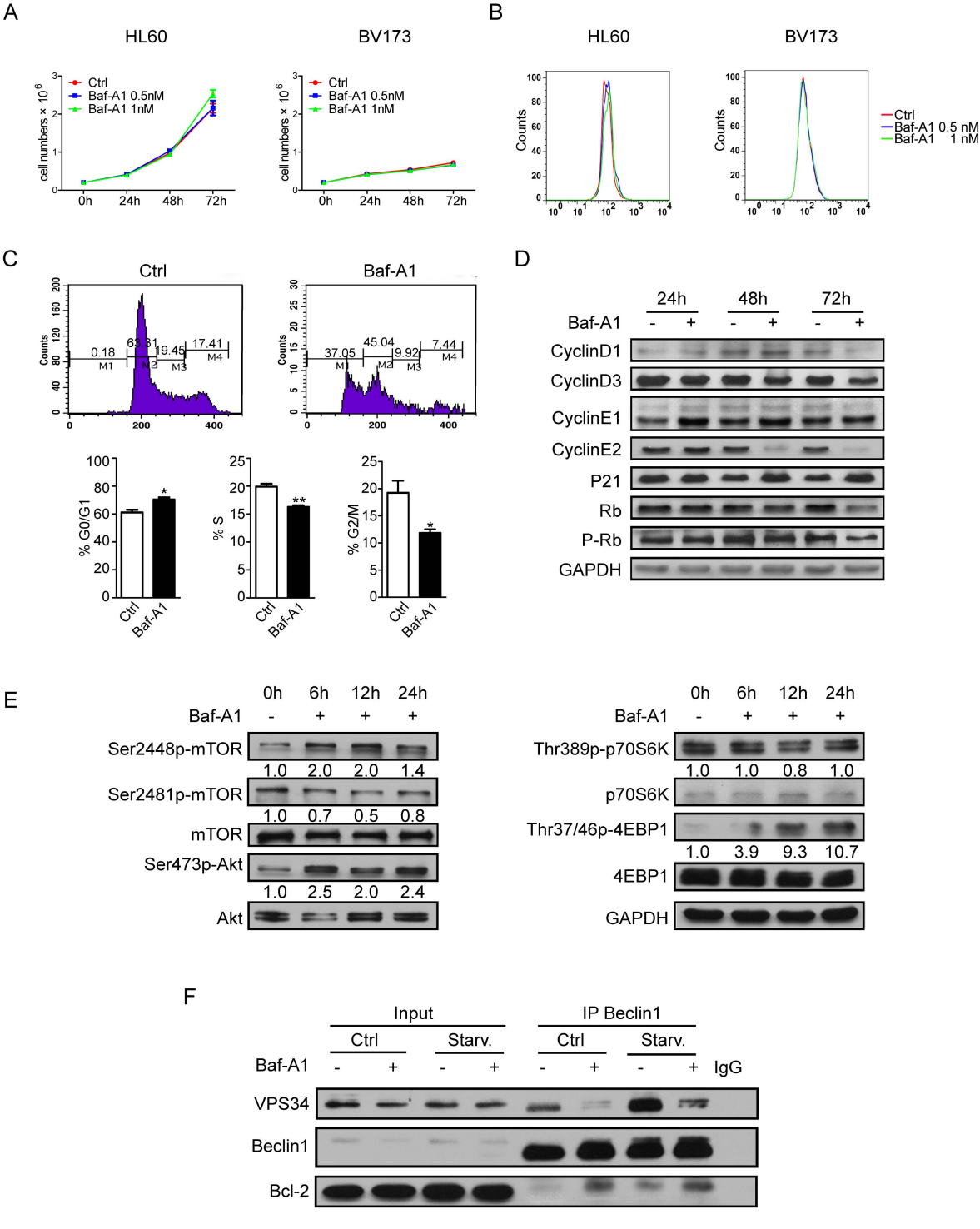
Analysis of mitochondrial membrane potential. The status of the mitochondrial membrane potential (ΔΨ) was evaluated by using the JC-1 probe (Molecular Probes, USA). Cells were stained with 10 μmol/L JC-1 for 15 min at 37℃ and then immediately transferred on ice and analyzed by flow cytometry.

Analysis of intracellular free Ca^{2+} concentration. Cells were cultured in six-well plates and pretreated with bafilomycin A1 (1 nM) for 72 h, Fluo-4/AM was used as an intracellular free Ca^{2+} fluorescent probe to analyze [Ca2+]i. Briefly, the harvested cells were incubated with Fluo-4/AM (5 m mol/L final concentration) for 30 min at 37℃ in the dark, washed with PBS, and analyzed on a BD Calibur flow cytometer. Intracellular [Ca2 +]i levels were represented by fluorescent intensity. Fluorescent intensity was recorded by excitation at 494 nm and emission at 516 nm. The data were analyzed by Cell Quest program (Becton Dickinson, USA), and the mean fluorescence intensity was obtained by histogram statistics.

Measurement of intracellular pH. After the treatment with 1 nM bafilomycin A1 for 72 h, B-ALL cells were washed and incubated with 1 μM LysoSensor Green DND-189 (Life Technologies, USA) for 30 min. Cells were then harvested, pelleted, and resuspended in PBS. Cells were kept on ice and protected from light during immediate transport to the BD Calibur flow cytometer for analysis.

Blood routine examination. 20 μl mouse peripheral blood was added into 500 μl CPK-303A solution (37℃), and blood routine examination was performed using Sysmex KX-21N (Sysmex, USA).

Supplementary Figures with Legends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Baf-A1 1nM
cell numbers × 10^6
0h
24h
48h
72h
B
HL60
BV173
Counts
Ctrl
Baf-A1 0.5 nM
Baf-A1 1 nM
C
Ctrl
Baf-A1
0.18
63.31
9.45
17.41
37.05
45.04
9.92
7.44
M1
M2
M3
M4
% G0/G1
% S
% G2/M
D
24h
48h
72h
Baf-A1
CyclinD1
CyclinD3
CyclinE1
CyclinE2
P21
Rb
P-Rb
GAPDH
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12h
24h
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mTOR
Ser473p-Akt
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Akt
Thr389p-p70S6K
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p70S6K
Thr37/46p-4EBP1
1.0 3.9 9.3 10.7
4EBP1
GAPDH
F
Input
IP Beclin1
Ctrl
Starv.
Baf-A1
IgG
VPS34
Beclin1
Bcl-2

Figure S1. Bafilomycin A1 preferentially inhibits pediatric B-ALL cells.

(**A**) Low concentration bafilomycin A1 treatment did not inhibit cell growth of AML HL-60 and CML BV173 cells as determined at 0, 24, 48 and 72 h of the treatment by MTT assay. (**B**) Low concentration bafilomycin A1 did not inhibit cell division of HL60 and BV173 cells as determined at 72 h of the treatment by flow cytometry with CFSE labeling. (**C**) Cell cycle analysis on B-ALL Nalm-6 cells. The Nalm-6 cells were treated with bafilomycin A1, and the cell cycle was analyzed by flow cytometry after PI staining. Bafilomycin A1 increased percentage of the cells in G0/G1 phase and decreased percentage of the cells in the S and G2/M phase of the cell cycle. The percentage of cells in each stage of the cell cycle after 72h of treatment as indicated is shown. Data are from three independent experiments. *: $p \leq 0.05$, **: $p \leq 0.01$. (**D**) Cell cycle regulators modulated by bafilomycin A1 in Nalm-6 cells. Nalm-6 cells were cultured with 1 nM Baf-A1, and cell cycle regulatory proteins were measured at 24, 48, and 72 h of treatment by western blotting. (**E**) Bafilomycin A1 activated mTOR signaling in Nalm-6 cells. The Nalm-6 cells were cultured with 1 nM Baf-A1 for the time course indicated. Akt, p-Akt, mTOR, p-mTOR and its downstream targets, p70S6K and 4EBP1, were measured by western blotting. The numbers below the blots indicate the relative intensity of phosphorylation-specific bands for mTOR, AKT, p70S6K and 4E-BP1 relative to total mTOR, AKT, p70S6K and 4E-BP1. (**F**) Co-immunoprecipitation of vps34 or Bcl-2 with Beclin1 as a bait of the Nalm-6 cells after either HBSS starvation or bafilomycin A1 treatment.

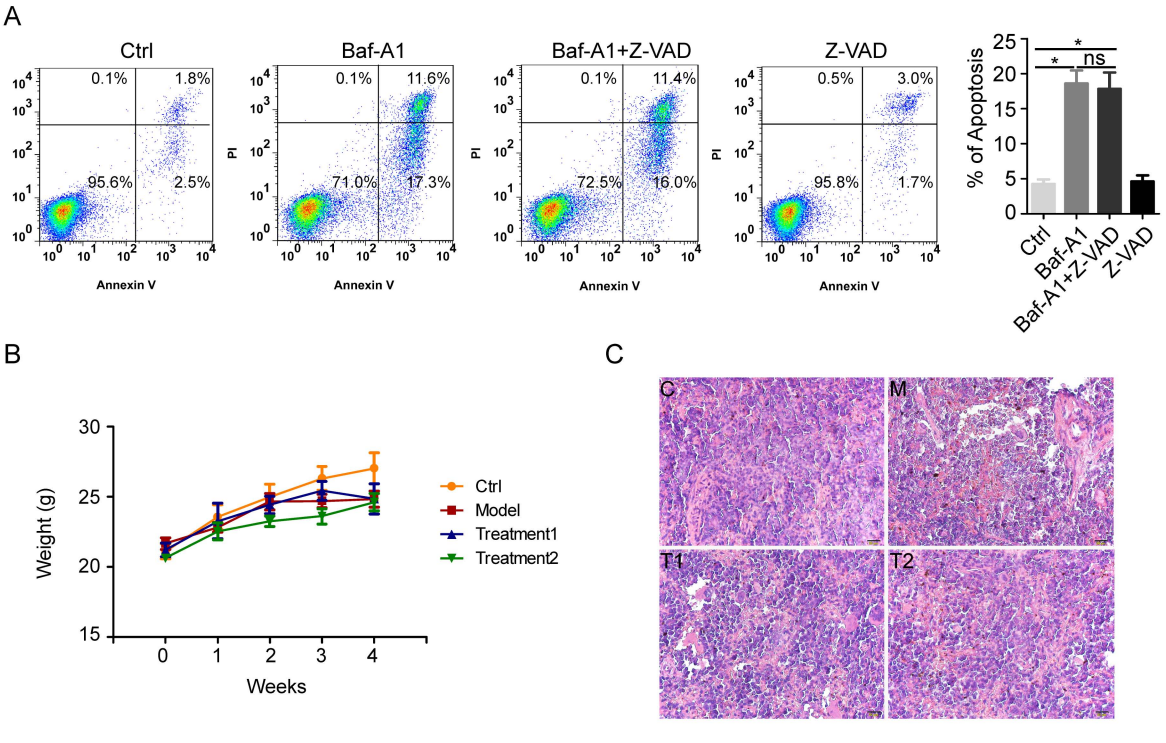
A
Ctrl
Baf-A1
Baf-A1+Z-VAD
Z-VAD
0.1%
1.8%
95.6%
2.5%
0.1%
11.6%
71.0%
17.3%
0.1%
11.4%
72.5%
16.0%
0.5%
3.0%
95.8%
1.7%
PI
Annexin V
% of Apoptosis
ns
Ctrl
Baf-A1
Baf-A1+Z-VAD
Z-VAD
B
Weight (g)
Weeks
Ctrl
Model
Treatment1
Treatment2
C
C
M
T1
T2

Figure S2. Bafilomycin A1 preferentially inhibits pediatric B-ALL cells.

(**A**) Bafilomycin A1 induced a caspase-independent apoptosis of Nalm-6 cells. The Nalm-6 cells were cultured with 1 nM bafilomycin A1 for 72 hours. Left, Flow cytometric analysis of Annexin V-FITC/PI stained Nalm-6 cells. Right, statistic data on bafilomycin A1-induced apoptosis level in Nalm-6 cells. Apoptosis was defined as the percentage of Annexin V–positive cells. Data are from three independent experiments. *: $p \leq 0.05$, **: $p \leq 0.01$. (**B**) Animal weights, monitored once a week over four weeks, displayed no difference between bafilomycin A1-treated groups and the control. (**C**) Hematoxylin and eosin–stained sections from spleen of the mice engrafted with B-ALL 697 cells, examined on day 30 after the completion of treatment with vehicle or bafilomycin A1.